

ExoPolySachharide Secreting Bacteria: Potential for Useful Applications

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ExoPolySachharide Secreting Bacteria, EPS, eukaryotic microbial cells, Microbial Production, Industrial Application of EPS, Usefulness of EPS

Abstract:

This research paper investigates into the usefulness of the ExoPolySachharide Secreting Bacteria. The research has been undertaken to explore further in the field of the potential uses of the ExoPolySachharide Secreting Bacteria.

It should be noted that over the years, only a few of the possible polysaccharide candidates such as cellulose, dextran, xanthan, levan, and alginate for commercial microbial production have been extensively studied. The search for new polysaccharides has resulted in the development of several new and useful applications with commercial potentials.

The research paper shows enormous potential of ExoPolySachharide Secreting Bacteria in isolation and characterization of such bacteria's from various available sources with a vision to find potential solutions to day-today problems of mankind associated with it.

INTRODUCTION:-

Exopolysaccharides (EPSs) are often found surrounding the outermost structures of both prokaryotic and eukaryotic microbial cells. They may be closely associated with the cell in the form of discrete capsules or else excreted as slime, unattached to the cell surface as such. EPSs exist in a wide variety of unique and often complex chemical structures and they are believed to provide self-protection against antimicrobial substances antibodies and bacteriophages and/or afford adherence to other bacteria, animal and plant tissues, or inert surfaces thus forming biofilms. They are also proving to have interesting bioactive functions and an extensive range of potential applications in industry, pharmacy, agriculture and various other areas. During the past 50 years a considerable number of bacterial EPSs have been described, but few have achieved great commercial success due either to their being unable to offer better properties than those already on the market or to difficulties in finding new practical applications. Actually, this industrially important biopolymers produced by microorganisms are exopolysaccharides that are secreted by bacteria outside their wall indicated by slime production. Mobility control in secondary and tertiary oil recovery, in petroleum drilling fluids and in the paint, pharmaceutical and cosmetic industries (*Aureobasidium*

pullulans is cultivated industrially for the production of useful polysaccharide, Pullulan.). Polysaccharide production in few bacteria has been shown to be controlled by plasmid (Muc+plasmid). Bacterial exopolysaccharides has been reported to help in biosorption of metal salt. *Arthrobacter viscosus* has found to accumulate more metal as compared to other strain that does not produce exopolysaccharide in the same way *Enterobacter* species was found to accumulate metals from culture medium. In the present study, we report the isolation and characterization of a soil bacterium capable of polysaccharide production. The polysaccharide contains mainly glucose and unidentified pentose. In addition to this the bacterium also accumulate in metal ions presence (Zinc, Iron etc.). The ability to produce exopolysaccharides (EPSs) with greatly varying composition and properties is a well-known biological phenomenon among bacteria. These EPSs are either secreted in the growth medium or remained attached to the bacterial cell wall as viscous, ropy or slime layers in growth medium. On the basis of monosaccharide composition in their backbone, bacterial EPSs have been classified into homopolysaccharides and heteropolysaccharides.

The majority of bacterial homopolysaccharides are neutral and synthesized outside the cell solely by the action of bacterial extracellular enzymes such as

dextranucrase and *levansucrase* which catalyze the production of dextran and levan, respectively. Whereas heteropolysaccharides are synthesized within the cell and then exported across the outer membrane or cell wall into the growth medium. Bacterial EPSs including most of the heteropolysaccharides are usually acidic (anionic) due to the presence of uranic acids or non-carbohydrate substituents such as pyruvate, sialic acid, succinate, lactic acid, phosphate, and carboxylate groups such as xanthan, alginate and succinoglycan etc. . To date, at least 20 different monosaccharides have been identified in reported bacterial EPSs. Among these, heteropolysaccharides are more common than homopolysaccharides and are very diverse because of copolymerization of various monomer units resulting in various possible types of linkages, various repeated monomers in chain and their relative combining ratios. Therefore, bacterial EPSs do not have an exact molecular weight and it is distributed over a broad range typically in 10³-10⁶ Da. This structural diversity is due to environmental and bacterial growth conditions and also depends on bacteria themselves .Physiologically, bacterial EPSs have been reported to play an important role in cell aggregation, cell adhesion, biofilm formation, water absorption and protect cells from hostile environmental conditions such as starvation, desiccation, phagocytosis and predation, etc. However, bacterial EPSs have been non-toxic,

biodegradable and environmental friendly .Further, bacterial EPSs offer a number of unique physical properties such as gelling, stabilizing, thickening, flocculating, and water absorbing and retaining, which have numerous commercial applications in food and non-food industries .Over the years, only a few of the possible polysaccharide candidates such as cellulose, dextran, xanthan, levan, and alginate for commercial microbial production have been extensively studied. The search for new polysaccharides has resulted in the development of several new and useful applications with commercial potentials.

Owing to these enormous potential in role of ExoPolySachharide Secreting Bacteria, we worked towards isolation and characterization of such bacteria's from various available sources with a vision to find potential solutions to day-today problems of mankind associated with it.



Some biofilms cause serious problems when they establish colonies inside Metal piping and hasten corrosion.

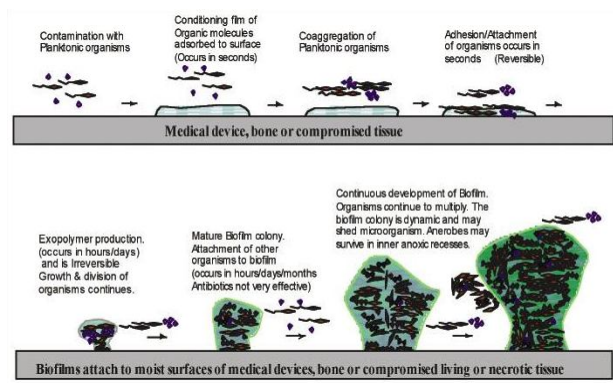


Fig.1 → Some biofilms cause serious problems when they establish colonies inside Metal piping and hasten corrosion.

Review Of literature:-

Exopolysaccharides are naturally formed macromolecules produced during the growing cycle of the organisms. Exopolysaccharides (EPSs) play important roles in the attachment of bacterial cells to a surface and in building and maintaining the three-dimensional, complex structure of bacterial biofilms (Laue et al., 2006). Their synthesis generally involves enzymatic catalysis, with chain increase through polymerization reactions of activated monomers, those being typically formed within the cells through complete metabolic processes (Flávia et al., 2006). One benefit of this environment is increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. In some cases antibiotic resistance can be increased 1000 fold (Stewart et al., 2001).

Exopolysaccharides may be either be homopolysaccharides or heteropolysaccharides. Of these some of the homopolysaccharides possess regular structures, but the dextrans and levans don't. Molecular masses of the EPS from lactic acid bacteria range from 10 kDa and over 200kDa to >1,000 kDa (Courtin et al., 2002). With the exception of bacterial alginates, the heteropolysaccharides are composed of regular repeat units of 2–8 monosaccharides (Mayer et al., 1999), enabling them to withstand considerable shear forces (Sutherland, 2001).

Importantly, both structure and molecular mass influence the rheological properties of a polysaccharide (Faber et al.1998) Also, environmental factors (carbohydrate source, nitrogen source, and carbon/nitrogen ratio of the growth medium) can influence the production, monomer composition, and molecular mass of the EPS produced by a particular strain (Degest and Vuyst, 1999). The structure and composition of the microbial polysaccharides depend on several factors, such as the composition of the culture medium, kind of carbon source, kind of microbial system utilized (aerobic or anaerobic), fermentation conditions (pH, temperature, oxygen concentrations) as well as the control. Low temperature favoured the increased production of EPS, the reason being that at low temperature the bacterial growth is slow but a

large amount of available sucrose is transformed to polymeric sugars i.e. exopolysaccharides (Tallgren et al., 1999). For the production of microbial gums, it is required to establish the optimal composition of the medium and the fermentation parameters. Several ions are added to the medium so as to provide the synthesis of exopolysaccharides, and those should be present in suitable amounts. Extracellular secreted sugar polymers, or EPS (exopolysaccharides) play an important role in development of texture of yogurts and other fermented milks, low fat cheeses and dairy desserts (Vuyst and Vaningelgem, 2003). Bacterial polysaccharides have indeed been shown to have the ability to bind cations, ion uptake and selectivity for some EPS being influenced by the level of acetylation (Geddie and Sutherland, 1993). These properties are potentially of great importance in sewage treatment processes for the removal of toxic heavy metal pollutants. In pathogenic situations, the presence of EPS can inhibit macrophage binding and also antibody coating, thereby blocking the immunological determinants required for opsonic phagocytosis (Hoiby, 1982). As Bacterial exopolysaccharide has been reported to help in bio-sorption of metal salts, therefore there is a possibility that the exopolysaccharide produced by the strain might be involved in metal accumulation. (Raihan Salman, Journal of Islamic Academy of Sciences

5:4, 282-285, 1992). Exopolysaccharide precipitates were observed after addition of 2 volumes of ethanol. Ethanol precipitation was done for all strains (Joo et al., 2004). The culture was diluted according to the growth and centrifugation was done to obtain cell pellet. The 2 volumes of ethanol were added to the supernatant. The EPS came to the upper layer of solvent. A slide was prepared taking small amount of EPS and much diluted little drop of crystal violet. This EPS when observed under microscope showed EPS fibers along with bacterial cells (Yuka et al., 1992). Many capsular strains of bacteria have been shown to produce loose slime in addition to capsules (Broadbent et al., 2003). The carbohydrate composition of EPS is unique to different strains of bacteria and may vary depending on the growth conditions; however, glucose and galactose in particular are frequently detected in the EPS composition of many bacterial species (Knoshaug et al., 2000). Although the growth limiting nutrient and the environmental conditions greatly affect EPS production. Temperature and pH have also been shown to affect the kinetics of polymer synthesis by several microorganisms (Zisu and Shah, 2003). Production of EPS is dependent on the temperature and pH of the medium as well as composition of the medium in terms of carbon and nitrogen source, and mineral and vitamin contents (Gorret et al., 2001). EPS generally

contains high molecular weight compounds with charged functional groups and possess both adsorptive and adhesive properties. Due to the presence of charged moieties, EPS ideally serves as a natural ligand source, providing binding sites for other charged particles/molecules including metals (Decho 1990). The metal binding properties of microbial EPS is well-studied (Mittelman and Geesey 1985, Geesey et al. 1988) and EPS is widely employed in bioremediation of heavy metals including Pb, Ni, Cd, etc. (Brown & Lester 1982, Loačc et al. 1997, Dong et al. 2000). The animals used the EPS as a source of energy and nutrition. The labile nature of the bacterial EPS and its ability to bind heavy metals might route the bound metals through the marine food chain, thereby transferring and aiding bioaccumulation of metal pollutants in the higher trophic animals (EPS: Marine Food chain's Heavy metal carrier,

V. Bhaskar and Narayan B Bhosle., NIO, Goa, 2006)

MATERIALS AND METHODS:

❖ Isolation and purification of bacterial culture :

Prospective samples for EPS production were collected from various regions in vicinity of Raipur region. Approximately 1 gm of sample was suspended in EPS Secreting Media. The

samples were then incubated at 28°C. The above medium is called as the "EPS Secreting Media", was used in all further experiments and tests omitting agar when appropriate. This sample was then incubated at 37°C for 48 hours. After incubation, swabbing was done on Nutrient Agar Plate and further incubated for 24 hours. Mucoid colonies were picked and re-streaked on another nutrient agar plate to obtain pure culture.

❖ Sample collection :

Samples were collected from two different sites i.e. non-contaminated and contaminated.

Non-contaminated sites were garden soil, infertile garden soil, fruit and vegetable market soil, pond soil (0 - 4 cm) and river soil (10 cm). While the contaminated sites were mixed sludge and sewage water contaminated soils. The samples were collected in sterilized tubes.

❖ Isolation and purification of bacterial strains:

Samples were incubated in nutrient broth for 24 h on shaking. Bacterial load was calculated by serial dilution method. The selected bacterial colonies were purified on nutrient agar plates and subsequently analysed for gram reaction and biochemical tests

Screening for Exopolysaccharide Production:



Figure 2. → Screening for EPS Production

Screening for exopolysaccharide production was done by inoculating bacterial growth in exopolysaccharide detection medium for 48 to 72 h. After incubation the exopolysaccharide produced by bacteria was precipitated by adding chilled Ethanol in the supernatant of broth. A fine network formed in upper layer of solvent showed exopolysaccharide and that comes to the top of the solvent layer.

❖ RESISTANCE TO HEAVY METALS:

Resistance to four different heavy metals like Zinc (Zn(II)), Copper(Cu (II)),Iron(Fe(III)) and lead(Pb(II)) were checked by incorporating different concentrations of metal salts to nutrient broth. Stock solutions were prepared by dissolving 0.10 gm of metal salt in 100ml of distilled water. Salt solutions were added after sterilization of media. For each of the EPS culture selected, prepared test tubes containing 50ml of nutrient broth medium and then add 400mg/100ml of the salt from each of the salt stock solution. Repeated the same procedure

using concentration as 800mg/100ml from the Stock Solution.

❖ GRAM STAINING:

An important laboratory technique to distinguish between two major bacterial groups, based on stain retention by their cell walls. Bacteria smears are fixed by flaming, then stained with crystal violet followed by iodine solution, and then rinsed with alcohol or acetone, decolorized, and counterstained with Safranin. The Gram-positive bacteria are stained bright purple or purple-black, while the gram-negative bacteria are pink. This staining technique is useful in bacterial taxonomy and identification and in indicating the fundamental differences in the cell-wall structure. Gram-negative bacteria lack peptidoglycan in the cell wall, while gram-positive bacteria have about 90 percent of their cell wall composed of peptidoglycan.

❖ WATER ABSORPTION CAPACITY:

The purified EPS was placed in the weighed empty tea bag and weighed again. This tea bag was placed in 20 ml distilled water in a beaker for 2 h then left for 1 h to drain. The drained tea bag was placed in a clean weighed beaker and weighed again. It was dried at 105 °C for 15 h and weighed again (Ando *et al.*, 1984).

Following formula was applied to get water absorption capacity.

(Water absorption capacity =

$$\frac{\{\text{Sample weight after absorption (g)} - \text{Sample weight before absorption (g)}\}}{\text{Dried sample weight (g)}}$$

❖ CARBOHYDRATE CONTENT ESTIMATION IN EPS :

❖ HEAVY METAL BINDING CAPACITY (via CU [II]) :

5ml. of polysaccharide solutions were put into tea bags and hanged on in flasks containing appropriate metal salt (here we utilised Cuorous Sulfate salt) and the setup was shaken at 20-40 rpm for 48 hrs. at 28°C. The quantity of metal removed from the solution, i.e. bound to the polymer was calculated by measuring ions in the solution at 0 hr. and 48 hr. by UV Spectrophotometry.

RESULTS AND DISCUSSION

Selection of EPS Secreting Media :-

S .No.	SAMPLE SOURCE CULTURE	EPS SECRETION	
		+	-
1	College garden soil		-
2	Fruit and vegetable market effluent	+	
3	Kota dairy effluent		-
4	Effluent of ice cream shop	++	
5	Under-construction site		-
6	Hospital waste disposal site (EPS I)	+++	
7	Stagnant muddy water from Campus Pond (EPS II)	++	
8	Mixture of different industrial effluents	+++	
9	Rotten fruits sample (EPS V)	+++	
10	Kitchen waste disposal waste	++	

- - means no EPS production

- + means some hazy material produced which might not be EPS

- ++ means some EPS production.

- +++ Excellent EPS Production.

Those samples which gave excellent EPS production (viz. EPS I, EPS II, EPS V) were selected and purified colonies so obtained were subject to characterization.



Fig. 3 → Soil samples cultured on Petri Plates for EPS Production



Fig. 4 → Control Inoculum for Characteristic Analysis

❖ HEAVY METAL ION RESISTANCE :

Samples which showed considerably good amount of EPS production were isolated and purified colonies were then prepared. Further, these purified colonies were subjected to heavy metal resistance test using metals Pb, Cu, Zn and Fe and the obtained results are tabulated below.



Fig. 5 → Heavy Metal Resistance Testing for selective EPS Strains & their Analysis

❖ HEAVY METAL RESISTANCE (400mg/ml):

SALT \ EPS	Pb	Zn	Cu	Fe
1	++	+++	+	-
2	++	+++	++	-
3	++	+++	++	-

❖ HEAVY METAL RESISTANCE (800mg/ml):

SALT \ EPS	Pb	Zn	Cu	Fe
1	+++	+++	-	-
2	++	+++	-	-
3	+++	+++	-	-

Thus metal resistance tests was successfully performed and Zinc was the most resisted metal by selective EPS strains.

GRAM STAINING:

The 3 samples which showed excellent EPS production were subjected to Gram’s staining phenomenon and the results obtained are tabulated below:-

<u>S.N</u> <u>O</u>	<u>BACTERI</u> <u>AL</u> <u>STRAIN</u>	<u>GRAM</u> <u>STAIN</u> <u>ING</u>	<u>SHAPE</u>
1	EPS I	Gram positive	BACILLUS
2	EPS II	Gram negative	COCCI
3	EPS V	Gram negative	ROD

WATER ABSORPTION CAPACITY:

S.No.	EPS STRAIN	Water Absorbed (in gm.)
1.	EPS I	1.638 gm
2.	EPS II	1.250gm
3.	EPS V	0.161gm

→EPS I and EPS II were found to contain high water absorbing capacity.

Table :- ‘Modified Phenol Sulphuric Acid Method

Test Tube No.	Standard Glucose (ml)	Distilled Water	Glucose Concentration	<u>Spectrophotometric</u> <u>Readings</u> <u>at λ=405</u>	
				I	II
				Blank	.0
1	.2	.8	40		
2	.4	.6	60		
3	.6	.4	80		
4	.8	.2	100		
5	.0	.0			
Unknown	.0				

DISCUSSION:-

The obtained Bacterial Colonies as isolated from various sources in vicinity, were found to secrete a large amount of EPS where different strains showed different results. The higher secreting one's were further studied and designated as EPS I, EPS II and EPS V in the given analysis, obtained under moderate Temperature(28°C), Pressure and pH(=7) conditions using EPS Detection Media. The Gram Staining Reaction results reveal both

Gram Positive and Gram Negative Strains, and these strains were found to be having 'Bacilli', 'Rod' and 'Cocci' Structure respectively. Also observed were their morphology where some were present as single cells, some were in chains and clusters of bacterial cells were also observed. Microorganisms produce EPS in two distinct forms: ropy EPS or loose slime that is excreted into surroundings and capsular EPS that remain adhered to cell surface creating a discrete covering. Many capsular strains of bacteria have been shown to produce loose slime in addition to capsules (Broadbent et. al, 2003). On ethanol precipitation, some strains showed thread like material whereas some showed suspended material in test tube of which the best were selected out.

Further resistance to four different heavy metals (viz. Pb, Zn, Cu, and Fe) were checked by incorporating different concentrations of metal salts to suitable Nutrient Broth media using EPS as control inoculum. The selected strains showed maximum resistance to Zinc (Zn) and Lead (Pb) which showed that these strains were obtained from bacteria that frequently faced more contamination with these metal salts.

The water absorbing capacity of three strains were also checked. The two strains out of these revealed good water absorbing capacity. Further steps were taken to spectrophotometrically analyse the total carbohydrate content in the given samples using modified Phenol Sulphuric Acid Method. The obtained good strains were further subjected to heavy metal ion detection using UV Spectrophotometric Analysis.

FUTURE PROSPECTS AND CONCLUSION

- Enhanced Oil Recovery (By Controlling Mobility).
- As a “Thickening Agent” in Food Products, Cosmetics, Paints Industry.
- Eco-friendly Sanitary Products.
- Bioremediation (By Biosorption of Metal Salts)

The field of biofilm research especially EPS secretion study, has traditionally been hindered by an inability to study the biofilm in non-destructive, three dimensional ways.

In addition, it has been difficult or impossible to assess gene expression and metabolism of the microbe at the single cell level within a biofilm. However, as a result of advances

in laser technology, digital imaging, scanning electron microscopy, and new fluorescent probes, researchers can now build a three dimensional model of biofilms and identify the location in the biofilm where the specific genes are being expressed. This broad-based initiative on microbial biofilms is designed to elucidate the mechanisms underlying their formation as well as development of strategies for the

prevention and treatment of microbial biofilm-associated diseases. Moreover, this initiative is intended to capitalize on contemporary research in immunology, microbiology, bio-engineering and computer technology that might synergize with current biofilm research.

Through careful control of culture conditions, through the use of enzymes or through use of mutants, it has proved possible to prepare microbial polysaccharides with altered structures. Mild chemical degradation procedures have also proved useful. It has also been noted that different microbial species or the strains may produce a range of poly saccharides with close structural similarities. A better understanding of the natural ecology of biofilms is required to assess how the costs and benefit of bacterial behaviors measured in the laboratory translate into fitness effects. A first step is to identify all the species and strains within natural biofilms, for which culture-independent techniques such as phylochips (DeSantis et al., 2007) and high-throughput sequencing show promise. Such coarse grained diversity measurements provide a useful first proxy of the potential for cooperation within bacterial groups: the higher the proportion of a single

strain within a biofilm, the more likely cooperative behavior can evolve and remain stable against exploitative mutants. Assaying diversity across time and space is also important, however, because strong initial competition may whittle down an initially diverse biofilm to a few species that then cooperate (Cascales et al., 2007).

In addition, cell lineages may partially segregate within biofilms simply because cell division and limited movement tend to produce clonal clusters (Kreft, 2004b; Xavier &

Foster, 2007). Under such conditions, the evolution of public good secretion may occur more easily and remain more stable than in mixed culture, where cooperative cells are readily susceptible to exploitation (Griffin et al., 2004; Diggle et al., 2007a; Sandoz et al., 2007).

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The three authors graduated from National Institute of Technology, Raipur and opted for biotechnology which is an emerging field of research and innovation. Rajesh Kumar Prasad decided to prepare for the coveted Civil Services, Rahul Gautam pursued higher studies and completed M. Tech from Surathkal while Sanket Behal decided to work in the industry to use his research oriented thinking and knowledge.

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